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(71) Applicant *(for all designated States except US)*: MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: STRADER, Catherine, D. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). CASCIERI, Margaret, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). MACNEIL, Douglas, J. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

(74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).

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(54) Title: MODIFIED NEUROPEPTIDE Y RECEPTORS

(57) Abstract

Modified neuropeptide Y receptors having deletions, replacements or additions in the third intracellular domain are identified and methods of making the modified receptors are provided. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, compounds identified through the use of the modified receptors, including modulators of the receptors, and the use of the compounds to treat conditions, including obesity, diabetes, anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac and cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, and Huntington's, Alzheimer's and Parkinson's diseases.

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TITLE OF THE INVENTION

MODIFIED NEUROPEPTIDE Y RECEPTORS

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of U.S. Serial No. 08/335,017, filed November 7, 1994, the contents of which are hereby incorporated by reference.

Neuropeptide Y (NPY) is a 36 residue, amidated peptide. It is anatomically co-distributed and co-released with norepinephrine in and from sympathetic postganglionic neurons ([1], [2], [3], [4], [5], [6]). Stimulation of the sympathetic nervous system under physiological circumstances such as exercise ([7], [8]) or exposure to the cold ([9], [10]) promotes an elevation of both norepinephrine and NPY.

NPY is believed to act in the regulation of appetite control ([11], [12]) and vascular smooth muscle tone ([13], [14]) as well as regulation of blood pressure ([6], [15], [16], [17]). NPY also decreases cardiac contractility ([18], [19], [20], [21], [22]). Congestive heart failure and cardiogenic shock are associated with probable releases of NPY into the blood ([23], [24], [25]). Regulation of NPY levels may be beneficial to these disease states [26].

At the cellular level, neuropeptide Y binds to a G-protein coupled receptor ([27], [28], [29], [30]). Neuropeptide Y is involved in regulating eating behavior and is an extremely potent orixigenic agent ([11], [12], [31]). When administered intracerebroventricularly or injected into the hypothalamic paraventricular nucleus (PVN) it elicits eating in satiated rats ([32], [33], [34]) and intraventricular injection of antisera to NPY decreases eating ([11], [31]). It has been shown to stimulate appetite in a variety of species and at different stages of development ([12]). Other effects on energy metabolism include decreased thermogenesis, body temperature and uncoupling protein, and increased white fat storage and lipoprotein lipase activity ([9], [35], [36], [37], [38], [39]). NPY levels in the PVN increase upon fasting ([40], [41], [42], [43], [44]), before a scheduled meal ([31], [36], [40]), and in both streptozotocin-induced and spontaneous diabetes ([36], [45], [46], [47], [48], [49]). Also, NPY levels are increased in genetically obese

and hyperphagic Zucker rats ([36], [50], [51]). Thus, a specific centrally acting antagonist for the appropriate NPY receptor subtype may be therapeutically useful for treating obesity and diabetes. Other disorders which might be targeted therapeutically include anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac and cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, and Huntington's, Alzheimer's and Parkinson's diseases ([26], [52]).

At least four receptor subtypes of the NPY family have been proposed based on pharmacological and physiological properties. The Y1 receptor is stimulated by NPY or PYY (peptide YY) and appears to be the major vascular receptor ([16], [53], [54], [55]). The Y2 receptor is stimulated by C-terminal fragments of NPY or PYY and is abundantly expressed both centrally and peripherally ([55], [56], [57], [58]). A third receptor (Y3) is exclusively responsive to NPY and is likely present in adrenal medulla, heart, and brain stem ([27], [59]). In addition, other subtypes of this receptor family are known to exist, based on pharmacological and physiological characterization ([60], [61], [62], [63]). The feeding behavior is stimulated potently by NPY, NPY_{2-36} and the Y1 agonist [Leu31, Pro34]NPY, but is not stimulated by the Y2 agonist NPY_{13-36} ([11], [64], [65], [66]). This pharmacology is not characteristic of the defined Y1, Y2 or Y3 receptors and can thus be attributed to a unique receptor, termed "atypical Y1" ([11], [65], [66]), that is responsible for evoking the feeding response. In addition, data indicate the existence of additional members of this receptor family including one subtype specific for peptide PP ([62], [63]), one with affinity for short C-terminal fragments of NPY which induce hypotension when administered systemically ([15], [17], [30], [67], [68]), and one associated with binding of NPY and PYY to brain sigma and phencyclidine binding sites ([61]).

The DNA encoding the Y1 receptor has been cloned and shown to be a G protein coupled receptor ([53], [69], [70]). G-protein coupled receptors are well-known to share substantial sequence homology to each other (71). Recently, DNA encoding the Y4 receptor

has been isolated using Y1 DNA probes [72]. In addition, DNA encoding the Y2 receptor has been isolated by expression cloning ([73], [74]). The cDNAs encoding these receptors are at least 45% identical at the DNA level and 30% at the protein level. Other NPY receptors have not been cloned.

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SUMMARY OF THE INVENTION

Modified neuropeptide Y receptors having deletions, replacements or additions in the third intracellular domain are identified and methods of making the modified receptors are provided. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, compounds identified through the use of the modified receptors, including modulators of the receptors, and the use of the compounds to treat conditions, including obesity, diabetes, anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac and

cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, and Huntington's, Alzheimer's and Parkinson's diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic diagram of G-protein signal transduction system. The receptor is shown as a seven-helical bundle. α, β, and γ indicate the three subunits of the G protein. E indicates an effector enzyme, such as adenylyl cyclase. The agonist (A) binding with high affinity to the receptor-G protein complex and with low affinity to the receptor alone is shown.

Figure 2. Schematic diagram of the hamster β_2 adrenergic receptor. The third intracellular loop comprises residues 221-273. The proximal and distal segments of this loop are drawn in cylinders.

Figure 3 shows the amino acid sequence of the human NPY1 receptor subtype aligned with that of the hamster β2-adrenergic receptor. The transmembrane helices are underlined.

DETAILED DESCRIPTION OF THE INVENTION

Modified neuropeptide Y receptors having deletions, replacements or additions in the third intracellular domain are identified and methods of making the modified receptors are provided. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, compounds identified through the use of the modified receptors, including modulators of the receptors, and the use of the compounds to treat conditions, including obesity, diabetes, anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac and cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, and Huntington's, Alzheimer's and Parkinson's diseases. Modulators, as described herein, include but are not limited to agonists, antagonists, suppressors and inducers.

Neuropeptide Y receptors belong to a class of receptors known as "G-protein coupled receptors." The term "G-protein coupled receptor" refers to any receptor protein that mediates its endogenous signal transduction through activation of one or more guanine nucleotide binding regulatory proteins (G-proteins). These receptors share common structural features, including seven hydrophobic transmembrane domains. G-protein coupled receptors include receptors that bind to small biogenic amines, including but not limited to beta-adrenergic receptors (βAR), alpha-adrenergic receptors (αAR) and muscarinic receptors, as well as receptors whose endogenous ligands are peptides, such as neurokinin, neuropeptide Y and glucagon receptors. Examples of βAR include beta-1, beta-2, and beta-3 adrenergic receptors.

G-protein coupled receptors are cell surface proteins that mediate the responses of a cell to a variety of environmental signals. Upon binding an agonist, the receptor interacts with one or more specific G proteins, which in turn regulate the activities of specific effector proteins. By this means, activation of G-protein coupled receptors amplifies the effects of the environmental signal and initiates a cascade of intracellular events that ultimately leads to defined cellular responses. G-protein coupled receptors function as a complex information processing network within the plasma membrane of the cell, acting to coordinate a cell's response to multiple environmental signals.

G-protein coupled receptors are characterized by the ability of agonists to promote the formation of a high affinity ternary complex between the agonist, the receptor and the G-protein (Figure 1). The α-subunit of the G protein contains a guanine nucleotide binding site which, in the high affinity ternary [G protein-receptor-agonist] complex, is occupied by GDP. In the presence of physiological concentrations of GTP, the GDP molecule in the guanine nucleotide binding site of the G protein is displaced by a GTP molecule. The binding of GTP dissociates the α subunit of the G protein from its ß and γ subunits and from the receptor, thereby activating the G-protein to

stimulate downstream effectors (adenylyl cyclase in the case of the β-adrenergic receptor (βAR)) and propagating the intracellular signal. Thus, the ternary complex is transient in the presence of physiological concentrations of GTP. Because the affinity of the agonist for the receptor-G protein complex is higher than its affinity for the uncomplexed receptor, one consequence of the destabilization of the ternary complex is a reduction in the affinity of the receptor for the agonist. Thus, the affinity of agonists for G-protein coupled receptors is a function of the efficiency with which the receptor is coupled to the G-protein. In contrast, antagonists bind with the same affinity to the receptor in the presence or absence of G-protein coupling.

The observation that agonist affinity can be reduced by conditions under which a receptor is not optimally coupled to its G-protein has important implications for the identification of agonists of G-protein coupled receptors, particularly identification based on ligand binding. If a receptor is not optimally coupled to the G-protein under the conditions of binding assays, an agonist will bind to the receptor with relatively low affinity. Thus, a screen that relies on a binding assay based on displacement of a radiolabeled ligand, although attractive for its ease and the potential for high throughput, poses the risk that a promising partial agonist might be overlooked because the agonist would bind predominantly to the low affinity state of the receptor, and thus would have low affinity in the binding assay. Consequently, functional assays are frequently used to screen for agonists of G-protein coupled receptors. However, functional assays (ranging from ex vivo muscle contraction assays to determination of second messenger levels in cells expressing exogenous cloned G-protein coupled receptors) are tedious and more time-consuming than ligand binding assays, and hence are not readily adapted to high-throughput screens. Because the modified receptors of the present invention bind agonists with high affinity in the presence or absence of the G-protein, they can be used in high throughput radioligand binding assays to screen for high affinity ligands, regardless of whether the ligands are agonists or antagonists.

G-protein coupled receptors consist of seven hydrophobic domains connecting eight hydrophilic domains. The hydrophobicity or hydrophilicity of the domains may be determined by standard hydropathy profiles, such as Kyte-Doolittle analysis (Kyte, J. and Doolittle, R.J.F. *J. Mol. Biol.* 157: 105 (1982)). The receptors are thought to be oriented in the plasma membrane of the cell such that the N-terminus of the receptor faces the extracellular space and the C-terminus of the receptor faces the cytoplasm, so that each of the hydrophobic domains crosses the plasma membrane. The receptors have been modeled and the putative boundaries of the extracellular, transmembrane and intracellular domains are generally agreed (for a review, see Baldwin, EMBO J. 12:1693, 1993). In general, the transmembrane domains are comprised of stretches of 20-25 amino acids in which most of the amino acid residues have hydrophobic side chains (including cysteine, methionine, phenylalanine, tyrosine, tryptophan, proline, glycine, alanine, valine, leucine, isoleucine), whereas the intracellular and extracellular loops are defined by contiguous stretches of several amino acids that have hydrophilic or polar side chains (including aspartate, glutamate, asparagine, glutamine, serine, threonine, histidine, lysine, and arginine). Polar amino acids, especially uncharged ones (such as serine, threonine, asparagine, and glutamine) are found in both transmembrane and extramembrane regions.

The extramembrane regions are characterized by contiguous stretches of three or more hydrophilic residues. In contrast, hydrophilic residues are found only in groups of 1-2, surrounded by hydrophobic residues, in the transmembrane domain. Thus, the transmembrane and extramembrane regions can be identified by the number of contiguous hydrophilic or hydrophobic amino acids in the primary sequence of the receptor, in addition to the constraints on the length of the hydrophobic segments given above. The boundaries between the transmembrane and extramembrane regions are often defined by the presence of charged or polar residues at the beginning or end of a stretch of hydrophobic amino acids. The locations of the

mutations in the receptors of the present invention are described on the basis of these models and can be specifically defined by the specific amino acid numbers of the residues being mutated.

By these criteria, the third intracellular loop is defined as the hydrophilic loop connecting the hydrophobic, putative transmembrane domains V and VI. For example, in hamster β_2 adrenergic receptor, the third intracellular loop would refer to amino acids 221 through 273. In accordance with the principles described above, the beginning of this loop is defined by the presence of Arg221 (a charged residue at the end of the hydrophobic stretch of residues 198-220) and Lys273 (a charged residue at the beginning of the hydrophobic stretch of residues 274-298). In the human NPY1 receptor (PCT International Application Publication Nos. WO93/09227 published 13 May 1993 and WO93/24515 published 9 December 1993, the contents of both of which are hereby incorporated by reference), the third intracellular loop refers to amino acids #233-260 (Figure 3). In accordance with the principles described above, the beginning of this loop is defined by the presence of Lys233 (a charged residue at the end of the long stretch of hydrophobic residues comprising helix 5) and Arg260 (a charged residue at the beginning of the long stretch of hydrophobic residues comprising helix 6). In the rat NPY1 receptor, the third intracellular loop refers to amino acids #232-259 (Eva, C., *et al.*, FEBS Lett. 271:81, 1990). In accordance with the principles described above, the beginning of this loop is defined by the presence of Lys232 (a charged residue at the end of the long stretch of hydrophobic residues comprising helix 5) and Arg259 (a charged residue at the beginning of the long stretch of hydrophobic residues comprising helix 6). In the human and rat NPY2 receptors, the third intracellular loop refers to amino acids #241-268 (Gerald, C., *et al.*, PCT International Application Publication No. WO95/21245, the contents of which are hereby incorporated by reference). In accordance with the principles described above, the beginning of this loop is defined by the presence of Arg241 (a charged residue at the end of the long stretch of hydrophobic residues comprising helix 5) and Lys268 (a charged residue at the

beginning of the long stretch of hydrophobic residues comprising helix 6). In the human NPY4 receptor, the third intracellular loop refers to amino acids #236-263 (Bard, J.A., *et al.*, PCT International Application Publication No. WO95/17906, the contents of which are hereby incorporated by reference). In accordance with the principles described above, the beginning of this loop is defined by the presence of Arg236 (a charged residue at the end of the long stretch of hydrophobic residues comprising helix 5) and Gln263 (a polar residue at the beginning of the long stretch of hydrophobic residues comprising helix 6). In the rat NPY4 receptor, the third intracellular loop refers to amino acids #236-263 (Bard, J.A., *et al.*, PCT International Application Publication No. WO95/17906). In accordance with the principles described above, the beginning of this loop is defined by the presence of Arg236 (a charged residue at the end of the long stretch of hydrophobic residues comprising helix 5) and Arg263 (a charged residue at the beginning of the long stretch of hydrophobic residues comprising helix 6).

The present invention pertains to modified neuropeptide Y receptors having deletions, replacements or additions in the third intracellular domain. Methods of designing and making modified receptors are provided. The modified receptors are uncoupled from or are poorly coupled to their respective neuropeptides. However, these modified receptors bind agonists with high affinity in the absence of G protein coupling. Because of their high intrinsic affinity for agonists, these modified receptors may be used in high throughput binding assays to identify compounds that bind to the receptor with high affinity, regardless of whether these compounds are agonists or antagonists. The invention includes the DNA encoding the modified receptors, the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, substances identified through the use of the modified receptors including specific modulators of the modified receptors, and the use of these substances in treating diseases, including obesity, diabetes, cardiovascular, and neurological disorders. Modulators identified in this process are useful as therapeutic agents.

Modulators, as described herein, include but are not limited to agonists, antagonists, suppressors and inducers.

Modified receptors may include genetic variants, both natural and induced. Induced modified receptors may be derived by a variety of methods, including but not limited to, site-directed mutagenesis. Techniques for nucleic acid and protein manipulation are well-known in the art and are described generally in Methods in Enzymology and in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1989).

It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally-occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of a modified receptor is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of the modified receptor. The term "functional derivative" is intended to include the "fragments," "variants," "degenerate

variants," "analogs" and "homologues" or to "chemical derivatives" of modified receptors. The term "fragment" is meant to refer to any polypeptide subset of modified receptors. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire modified receptor molecule or to a fragment thereof. A molecule is "substantially similar" to a modified receptor if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical.

The term "analog" refers to a molecule substantially similar in function to either the entire modified receptor molecule or to a fragment thereof.

"Substantial homology" or "substantial similarity", when referring to nucleic acids means that the segments or their complementary strands, when optimally aligned and compared, are identical with appropriate nucleotide insertions or deletions, in at least 50% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize to a strand or its complement.

The nucleic acids claimed herein may be present in whole cells or in cell lysates or in a partially purified or substantially purified form. A nucleic acid is considered substantially purified when it is purified away from environmental contaminants. Thus, a nucleic acid sequence isolated from cells is considered to be substantially purified when purified from cellular components by standard methods while a chemically synthesized nucleic acid sequence is considered to be substantially purified when purified from its chemical precursors.

Nucleic acid compositions of this invention may be derived from genomic DNA or cDNA, prepared by synthesis or by a combination of techniques.

The natural or synthetic nucleic acids encoding the modified G-coupled protein receptors of the present invention may be

incorporated into expression vectors. Usually the expression vectors incorporating the modified receptors will be suitable for replication in a host. Examples of acceptable hosts include, but are not limited to, prokaryotic and eukaryotic cells.

The phrase "recombinant expression system" as used herein means a substantially homogenous culture of suitable host organisms that stably carry a recombinant expression vector. Examples of suitable hosts include, but are not limited to, bacteria, yeast, fungi, insect cells, plant cells and mammalian cells. Generally, cells of the expression system are the progeny of a single ancestral transformed cell.

The cloned modified receptor DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant modified receptor. Techniques for such manipulations are fully described in Sambrook, J., *et al.*, *supra*, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells, fungal cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungi or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes

mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant modified receptor in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant modified receptor expression, include but are not limited to, pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and λZD35 (ATCC 37565), pCI-neo (Promega).

A variety of bacterial expression vectors may be used to express recombinant modified receptor in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant modified receptor expression include, but are not limited to pET11a (Novagen), lambda gt11 (Invitrogen), pcDNAII (Invitrogen), pKK223-3 (Pharmacia).

A variety of fungal cell expression vectors may be used to express recombinant modified receptor in fungal cells. Commercially available fungal cell expression vectors which may be suitable for recombinant modified receptor expression include but are not limited to pYES2 (Invitrogen), *Pichia* expression vector (Invitrogen).

A variety of insect cell expression vectors may be used to express recombinant receptor in insect cells. Commercially available insect cell expression vectors which may be suitable for recombinant expression of modified receptor include but are not limited to pBlue Bac III (Invitrogen).

An expression vector containing DNA encoding modified receptor may be used for expression of modified receptor in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such

as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to Drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, lipofection, protoplast fusion, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce modified receptor protein. Identification of modified receptor expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-modified receptor antibodies.

Expression of modified receptor DNA may also be performed using *in vitro* produced synthetic mRNA or native mRNA. Synthetic mRNA or mRNA isolated from modified receptor producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being preferred.

The term "substantial homology", when referring to polypeptides, indicates that the polypeptide or protein in question exhibits at least about 30% homology with the naturally occurring protein in question, usually at least about 40% homology.

The modified receptors may be expressed in an appropriate host cell and used to discover compounds that affect the modified receptor. Preferably, the modified receptors are expressed in a mammalian cell line, including but not limited to, COS-7, CHO or L cells, or an insect cell line, including but not limited to, Sf9 and Sf21, and may be used to discover ligands that bind to the receptor and alter or stimulate its function. The modified receptors may also be produced in bacterial, fungal or yeast expression systems.

The expression of the modified receptor may be detected by use of a radiolabeled ligand specific for the receptor. For example, for the β_2 adrenergic receptor, such a ligand may be ^{125}I-iodocyanopindolol (^{125}I-CYP). For the NPY receptor, such a ligand may be ^{125}I-NPY, ^{125}I-Peptide YY (PYY) or ^{125}I-Pancreatic polypeptide.

The specificity of binding of compounds showing affinity for the modified receptors is shown by measuring the affinity of the compounds for cells transfected with the cloned modified receptor or for membranes from these cells. Expression of the cloned modified receptor and screening for compounds that inhibit the binding of radiolabeled ligand to these cells provides a rational way for rapid selection of compounds with high affinity for the receptor. These compounds may be agonists or antagonists of the receptor. Because the modified receptor does not couple well to G proteins, the agonist activity of these compounds is best assessed by using the wild-type receptor, either natively expressed in tissues or cloned and exogenously expressed.

Once the modified receptor is cloned and expressed in a mammalian cell line, such as COS-7 cells or CHO cells, the recombinant modified receptor is in a well-characterized environment. The membranes from the recombinant cells expressing the modified receptor are then isolated according to methods known in the art. The isolated membranes may be used in a variety of membrane-based receptor binding assays. Because the modified receptor has a high affinity for agonists, ligands (either agonists or antagonists) may be

identified by standard radioligand binding assays. These assays will measure the intrinsic affinity of the ligand for the receptor.

The present invention provides methods of generating modified NPY receptors. Such methods generally comprise the deletion of at least one nucleotide from the third intracellular domain of the receptor. Additional methods include, but are not limited to, enzymatic or chemical removal of amino acids from the third intracellular domain of the receptor. One method of generating modified NPY receptors comprises:

(a) isolating DNA encoding an NPY receptor;

(b) altering the DNA of step (a) by deleting at least one nucleotide from DNA encoding the third intracellular domain of the NPY receptor or disrupting the amphipathic helix at the N- or C-terminus of the third intracellular domain by replacement with nucleotides or addition of nucleotides coding for non-helical protein sequence;

(c) isolating the altered DNA;

(d) expressing the altered DNA; and

(e) recovering the modified NPY receptor.

The third intracellular domain of a G-protein coupled receptor is located between the fifth and sixth hydrophobic transmembrane domains of the receptor.

The present invention provides methods of identifying compounds that bind to modified NPY receptors. Methods of identifying compounds are exemplified by an assay, comprising:

a) cloning a neuropeptide Y receptor;

b) altering the DNA sequence encoding the third intracellular domain of the cloned receptor;

c) splicing the altered receptor into an expression vector to produce a construct such that the altered receptor is operably linked to transcription and translation signals sufficient to induce expression of the receptor upon introduction of the construct into a prokaryotic or eukaryotic cell;

d) introducing the construct into a prokaryotic or eukaryotic cell which does not express the altered receptor in the absence of the introduced construct; and

e) incubating cells or membranes isolated from cells produced in step c with a quantifiable compound known to bind to the receptors, and subsequently adding test compounds at a range of concentrations so as to compete the quantifiable compound from the receptor, such that an IC_{50} for the test compound is obtained as the concentration of test compound at which 50% of the quantifiable compound becomes displaced from the receptor.

The present invention is also directed to methods for screening for compounds which modulate the expression of DNA or RNA encoding modified receptors or which modulate the function of modified receptor protein. Compounds which modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding modified receptor, or the function of modified receptor protein. Compounds that modulate the expression of DNA or RNA encoding modified receptor or the function of modified receptor protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample.

Kits containing modified receptor DNA, antibodies to modified receptor, or modified receptor protein may be prepared. Such kits are used to detect DNA which hybridizes to modified receptor DNA or to detect the presence of modified receptor protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic, taxonomic or epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen

and measure levels of modified receptor DNA, modified receptor RNA or modified receptor protein. The recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of modified receptor. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant modified receptor protein or anti-modified receptor antibodies suitable for detecting modified receptor. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Pharmaceutically useful compositions comprising modulators of modified receptor activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such

moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations,

the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

The modified G-protein coupled receptors of the present invention are exemplified herein by the neuropeptide Y receptors.

Deletion mutagenesis of the β_2-adrenergic receptor has shown that none of the hydrophobic clusters of amino acids (the putative transmembrane helices) could be deleted without substantial loss of binding. In contrast, most of the connecting loops could be deleted without affecting the ligand binding properties of the receptor. This indicates that these hydrophilic loops are not required for ligand binding to the receptor, suggesting that the ligand binding pocket is located predominantly within the transmembrane domain of the protein (Strader, *et al. FASEB J* .3: 182-183 (1989)). Deletions in the connecting loops that were large enough to encompass the entire loop led to steric problems, resulting in incorrect processing of the protein (Dixon, *et al. EMBO J.* 6: 3269-3275 (1987)). Certain connecting loop deletion mutations, however, led to loss of functional activation of adenylyl cyclase by the receptor. For example, deletion of the carboxy terminal region of the third intracellular loop attenuated the ability of the receptor to activate adenylyl cyclase, and deletion of the amino terminal portion of this loop abolished adenylyl cyclase activation

(Strader, *et al. J. Biol. Chem.* 262: 16439-16443 (1987)). Moreover, the agonist binding isotherms for these modified receptors displayed a single affinity site, suggesting altered G protein interactions. Since these modified receptors also retain their functional activation of Na^+-H^+ exchange, which is mediated through a different G protein (Barber, *et al. Mol. Pharm.* 41: 1056-1060 (1992)), the deletions appear not to result in gross structural perturbations of the receptor, suggesting that the changes seen in adenylyl cyclase activation are due to alteration of a specific G protein interaction. Subsequent amino acid replacements in the third intracellular loop confirmed the role of this region in G protein interaction (Cheung, *et al. Mol. Pharm.* 41: 1061-1065 (1992)).

Modified NPY1 receptors lacking between 6 and 12 amino acids in the N terminal portion of the third intracellular loop (connecting transmembrane helices 5 and 6) may be synthesized. The bottom of transmembrane helix 5 is defined by the presence of a charged amino acid (human NPY1 Lys233, rat NPY1 Lys232) at the end of a series of hydrophobic amino acids. The modified receptors include the deletion of 6-12 residues following Lys233 (human) or Lys232 (rat) (i.e., I^{234}YIRLKRRNNMM; Seq. I.D. No. 1). Alternatively, this sequence could be disrupted by deletion of one or more of the charged residues (ie., K238, R239 or R240), or replacement of such residues with alanine or a helix-disrupting residue such as proline.

A second group of modified NPY1 receptors encompass the deletion of 6-13 residues at the C terminal end of the third intracellular loop of the receptor. The C terminus of this loop is defined by the bottom of helix 6, defined by the presence of the charged residue Arg260 (human NPY1) or Arg259 (rat NPY1) preceding a stretch of hydrophobic amino acids. The modified receptors of this group have deletions of 6-13 residues preceding Arg 260 in human NPY1 (i.e., KMRDNKYRSSETK^{259}; Seq. I.D. No. 2) and proceeding Arg259 in rat NPY1 (i.e., KIRDSKYRSSETK^{258}; Seq. I.D. No. 4). Alternatively, this sequence could be disrupted by deletion of one or more of the

charged residues (ie., R249, D250, K252), or replacement of such residues with alanine or a helix-disrupting residue such as proline.

Modified NPY2 receptors lacking between 6 and 12 amino acids in the N terminal portion of the third intracellular loop (connecting transmembrane helices 5 and 6) may be synthesized. The bottom of transmembrane helix 5 is defined by the presence of a charged amino acid (Arg241 in rat and human) at the end of a series of hydrophobic amino acids. The modified receptors include the deletion of 6-12 residues following Arg241 (i.e., I^{242}WSKLKNHVSPG; Seq. I.D. No. 5). Alternatively, this sequence could be disrupted by deletion of one or more of the charged residues (ie., K244, K246 or H248), or replacement of such residues with alanine or a helix-disrupting residue such as proline.

A second group of modified NPY2 receptors encompass the deletion of 6-13 residues at the C terminal end of the third intracellular loop of the receptor. The C terminus of this loop is defined by the bottom of helix 6, defined by the presence of the charged residue (Lys268 in human and rat) preceding a stretch of hydrophobic amino acids. The modified receptors of this group have deletions of 6-13 residues preceding Lys268 in human NPY2 (i.e., ANDHYHQRRQKTT^{267}; Seq. I.D. No. 6) and proceeding Lys268 in rat NPY2 (i.e., AASDHYHQRRHKTT^{267}; Seq. I.D. No. 7). Alternatively, this sequence could be disrupted by deletion of one or more of the charged residues (ie., D257, H258, H260, R262, R263, H264, K265), or replacement of such residues with alanine or a helix-disrupting residue such as proline.

Modified NPY4 receptors lacking between 6 and 12 amino acids in the N terminal portion of the third intracellular loop (connecting transmembrane helices 5 and 6) may be synthesized. The bottom of transmembrane helix 5 is defined by the presence of a charged amino acid (Arg236 in rat and human) at the end of a series of hydrophobic amino acids. The modified receptors include the deletion of 6-12 residues following Arg236 (i.e., I^{237}YRRLQRQGRVF in human NPY4 (Seq. I.D. No. 8) and I^{237}YQRLQRQRRAF in rat NPY4

(Seq. I.D. No. 9)). Alternatively, this sequence could be disrupted by deletion of one or more of the charged residues (ie., R238, R239, R242, R245), or replacement of such residues with alanine or a helix-disrupting residue such as proline.

A second group of modified NPY4 receptors encompass the deletion of 6-13 residues at the C terminal end of the third intracellular loop of the receptor. The C terminus of this loop is defined by the bottom of helix 6, defined by the presence of the charged residue (Gln263 in human and Arg263 in rat) preceding a stretch of hydrophobic amino acids. The modified receptors of this group have deletions of 6-13 residues preceding Gln263 in human NPY4 (i.e., HKGTYSLRAGHMK^{263}; Seq. I.D. No. 10) and proceeding Arg263 in rat NPY4 (i.e., HTHTCSSRVGQMK^{263}; Seq. I.D. No. 11). Alternatively, this sequence could be disrupted by deletion of one or more of the charged residues (ie., H251, K252, R258, H261), or replacement of such residues with alanine or a helix-disrupting residue such as proline.

Other modified receptors encompass the deletion of 6-13 residues at either the N or C terminal end of the third intracellular loop, or replacement of residues within this region, of other members of the family of NPY receptors. The N terminus of the third intracellular loop (connecting transmembrane helices 5 and 6) is defined by the presence of a charged or polar amino acid at the end of the fifth series of hydrophobic amino acids in the sequence of the receptor (helix 5). The C terminus of this loop is located at the bottom of helix 6, defined by the presence of a charged or polar residue preceding the sixth stretch of hydrophobic amino acids.

Other modified receptors encompass the addition of 5 to 10 residues at either the N or C terminal end of the third intracellular loop of the NPY1, 2 or 4 receptors such that the amphipathic nature of these regions is disrupted.

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of the examples.

EXAMPLE 1

Deletion of 6-13 amino acids at the N-terminal portion of the third intracellular loop of the human Neuropeptide Y1 receptor

Modified receptor is constructed by site-directed mutagenesis of the human neuropeptide Y1 receptor cDNA by standard molecular biological techniques.

The modified DNA sequence encodes a human neuropepide Y1 receptor lacking between 6 and 13 amino acid residues at the N-terminal portion of the third intracellular loop. The nucleotide sequence of the modified receptor is confirmed by DNA sequencing. As with modified β_2 receptors, the modified NPY receptor is designed so as to disrupt the proximal portion of the third intracellular loop, without affecting the adjacent fifth transmembrane helix. Thus, the charged amino acid that delineates the bottom of helix 5 (Lys233) is left intact in the modified receptor, while the six to thirteen amino acids which follow it are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, beginning immediately after the charged residue at the bottom of transmembrane helix 5, for example D(234-241)NPY1 receptor.

EXAMPLE 2

Deletion of 6-13 amino acids at the C-terminal portion of the third intracellular loop of the human Neuropeptide Y1 receptor

Modified human NPY1 receptor, lacking 13 residues at the C-terminal portion of the third intracellular loop (D(247-259)NPY1 receptor), is prepared by standard mutagenesis procedures. The nucleotide sequences of the modified receptors are confirmed by DNA sequencing. This modified human NPY1 receptor is designed so as to disrupt the distal portion of the third intracellular loop, without affecting the adjacent sixth transmembrane helix. Thus, the charged amino acid that defines the bottom of helix 6 (Lys260) is left intact,

while the nearby proximal residues are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, ending immediately before the charged residues at the bottom of helix 6.

EXAMPLE 3

Expression and characterization of the altered Neuropeptide Y1 receptor

COS-7 cells are transfected with the modified receptor cDNA subcloned into a eukaryotic expression vector such as the eukaryotic expression vector pcDNA I/neo (Invitrogen). Cells are harvested after incubation for about 60-72 h. Membranes containing the expressed receptor protein are prepared as described (C. D. Strader *et al., Proc. Natl. Acad. Sci. U.S.A.* 84, 4384-4388 (1987).

Binding reactions are performed in a final volume of 250 µl of buffer A (50 µM Tris, pH 7.4 containing 20 mM $CaCl_2$, 5 mM KCl, 0.2% bovine serum albumin, 10 µM phosphoramidon, 40 µg/ml bacitracin and 2 µg/ml leupeptin). ^{125}I-NPY or ^{125}I-PYY (0.1 nM) is incubated with membranes for 2 hr at 25°C before filtration over GF/C filters presoaked in 0.1% polyethyleneimine. Filters are washed with ice cold buffer A before analysis of the bound radioactivity by γ scintillation counting.

Membranes prepared from the COS-7 cells transfected with a vector containing either the wild type or the modified receptor cDNA specifically bind a radiolabeled neuropeptide Y receptor radioligand. The modified receptor is characterized by an absence of coupling to G proteins, an inability to mediate the activation of second messenger systems, and an increased affinity for agonists.

The modified neuropeptide Y receptor, when expressed in mammalian cells, does not stimulate G protein activation in response to the agonist NPY. In contrast, when the wild type receptor is expressed in the same cell line, activity is stimulated.

These modified receptors have increased affinity for agonists when compared to the wild type receptor. The wild type NPY1 receptor can be described pharmacologically by the relative potency of peptide ligands: neuropeptide Y= peptide YY > [Leu31Pro34]NPY > NPY[2-36]>> NPY[13-36], with the affinity of NPY in the range of 0.1-10 nM, and NPY[13-36] having an affinity in the μM range. The mutant receptor binds the agonists with the same relative order of potency. The high affinity of the agonist for the modified receptor is not affected by agents that uncouple the receptor from the G protein; such agents include the nonhydrolyzable GTP analog GppNHp, sodium fluoride, and the detergent digitonin. In contrast, the wild type receptor binds agonists with two affinity states: a high affinity state, indicative of binding to the receptor-G protein complex, and a low affinity state, reflecting binding to the uncoupled receptor alone. When the receptor is not optimally coupled to the G protein, a binding assay using the modified receptor will detect agonists with more sensitivity than will the identical binding assay using the wild-type receptor.

EXAMPLE 4

Screening Assay using modified Neuropeptide Y1 receptors

Transfected cells expressing recombinant modified receptor may be used to identify compounds that bind to the receptor with high affinity. This may be accomplished in a variety of ways, such as by incubating the test compound in a final volume of 0.25 ml of buffer A with membranes containing 5-7 pM of the modified neuropeptide Y receptor and 100 pM ^{125}I-PYY or ^{125}I-NPY for 2 hour at 25°. The reaction is stopped by filtration over GF/C glass fiber filters presoaked in 0.1% polyethyleneimine, washing with 3 x 5 ml of cold buffer A, and counting the filters in a gamma counter to measure bound radioactivity. This assay will detect a compound that has a high intrinsic affinity for the receptor. Such compounds may be either agonists or antagonists.

EXAMPLE 5

Deletion of 6-13 amino acids at the N-terminal portion of the third intracellular loop of Neuropeptide Y receptor subtypes

Modified NPY receptor subtypes (e.g., NPY2, NPY4) having deletions at the N terminal region of the third intracellular loop are constructed by site-directed mutagenesis of the neuropeptide Y receptor cDNA by standard molecular biological techniques.

The modified DNA sequence encodes a neuropepide Y receptor lacking between 6 and 13 amino acid residues at the N-terminal portion of the third intracellular loop. The nucleotide sequence of the modified receptor is confirmed by DNA sequencing. The modified NPY receptor is designed so as to disrupt the proximal portion of the third intracellular loop, without affecting the adjacent fifth transmembrane helix. Thus, the charged amino acid that delineates the bottom of helix 5 is left intact in the modified receptor, while the six to thirteen amino acids which follow it are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, beginning immediately after the charged residue at the bottom of transmembrane helix 5.

EXAMPLE 6

Deletion of 6-13 amino acids at the C-terminal portion of the third intracellular loop of the Neuropeptide Y receptor

Modified NPY receptor subtypes (e.g., NPY2, NPY4) having deletions at the C terminal region of the third intracellular loop are constructed by site-directed mutagenesis of the neuropeptide Y receptor cDNA by standard molecular biological techniques. The nucleotide sequences of the modified receptors are confirmed by DNA sequencing. These modified NPY receptors have disruptions in the distal portion of the third intracellular loop, without affecting the adjacent sixth transmembrane helix. Thus, the polar amino acid that defines the bottom of helix 6 is left intact, while the nearby proximal

residues are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, ending immediately before the polar residues at the bottom of helix 6.

EXAMPLE 7

Expression and characterization of modified NPY Receptor

The modified receptor is subcloned into an expression vector such as pRC/CMV (Invitrogen,San Diego, CA) and expressed in mammalian cells by transfection. Approximately 72 hours after transfection, cells are harvested for radioligand binding assays.

For binding assays, the membranes are prepared by harvesting the cells in ice-cold lysis buffer (5 mg Tris, pH 7.4; 2 mM EDTA), followed by 15 min centrifugation at 38,000 x g. The membrane pellet is then resuspended in buffer A. Equilibrium binding to the wild type or modified NPY receptor is performed in a final volume of 0.25 ml containing membranes, 100 pM ^{125}I-PYY, and serial dilution of the competing ligands. Binding reactions are incubated for 2 hr at 25°C, and terminated by rapid filtration over GF/C filters pre-soaked in 0.1% polyethyleneimine. The radioactivity is quantified with a Packard gamma counter.

These modified receptors have increased affinity for agonists when compared to the wild type receptor. The wild type "atypical NPY1" or NPY4 receptor that mediates feeding behavior can be described pharmacologically by the high affinity of neuropeptide Y, peptide YY, NPY[2-36], and [Leu31Pro34]NPY, and the lower affinity of more truncated analogs NPY[13-36] and NPY [20-36], and structurally by its sequence homology (>45% at the DNA level) to the NPY1 receptor. The affinity of NPY for the atypical Y1 receptor subtype is in the range of 0.01-10 nM, and that for NPY[13-36] is in the 0.1-10 μM range. The mutant receptor binds the agonists with the same relative order of potency as the wild type receptor. The high affinity of the agonist for the modified receptor is not affected by agents that uncouple the receptor from the G protein; such agents include the nonhydrolyzable GTP analog GppNHp, sodium fluoride, and the

detergent digitonin. In contrast, the wild type receptor binds agonists with two affinity states: a high affinity state, indicative of binding to the receptor-G protein complex, and a low affinity state, reflecting binding to the uncoupled receptor alone. When the receptor is not optimally coupled to the G protein, a binding assay using the modified receptor will detect agonists with more sensitivity than will the identical binding assay using the wild-type receptor. Other NPY receptor subtypes (NPY2, NPY3, and others) are also defined pharmacologically by the relative potencies of peptide ligands for these receptors and structurally by their sequence similarity to the NPY 1 receptor. Mutant receptors having deletions in the third intracellular loop have similar orders of potency as the corresponding wild type receptor, but with higher affinity than the wild type receptor in the absence of G protein coupling.

These modified NPY receptors are readily used in a screening assay to detect compounds that bind with high affinity to the NPY receptor subtype, regardless of whether these compounds are agonists or antagonists.

EXAMPLE 8

Cloning and Expression of Modified NPY Receptor cDNA into Bacterial Expression Vectors

Recombinant modified receptor is produced in a bacterial expression system such as E. coli. The modified receptor expression cassette is transferred into an E. coli expression vector; expression vectors include but are not limited to, the pET series (Novagen). The pET vectors place modified receptor expression under control of the tightly regulated bacteriophage T7 promoter. Following transfer of this construct into an E. coli host which contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of modified receptor is induced by addition of an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed modified receptor are determined by the assays described herein.

EXAMPLE 9

Cloning and Expression of Modified NPY Receptor cDNA into a Vector for Expression in Insect Cells

Baculovirus vectors derived from the genome of the AcNPV virus are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculovirus expressing modified receptor cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the modified receptor cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P.A., *Nuc. Acid. Res.* 18, 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of β-galactosidase expression (Summers, M. D. and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, modified receptor expression is measured.

Authentic modified receptor is found in association with the infected cells. Active modified receptor is extracted from infected cells by hypotonic or detergent lysis.

Alternatively, the modified receptor is expressed in the *Drosophila* Schneider 2 cell line by cotransfection of the Schneider 2 cells with a vector containing the modified receptor DNA downstream and under control of an inducible metallothionin promoter, and a vector encoding the G418 resistant neomycin gene. Following growth in the presence of G418, resistant cells are obtained and induced to express modified receptor by the addition of $CuSO_4$. Identification of modulators of the modified receptor is accomplished by assays using either whole cells or membrane preparations.

EXAMPLE 10

Cloning of Modified NPY Receptor cDNA into a yeast expression vector

Recombinant modified receptor is produced in the yeast S. cerevisiae following the insertion of the modified receptor cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of heterologous proteins. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the modified receptor cistron [Rinas, U. *et al.*, *Biotechnology* 8, 543-545 (1990); Horowitz B. *et al.*, *J. Biol. Chem.* 265, 4189-4192 (1989)]. For extracellular expression, the modified receptor cistron is ligated into yeast expression vectors which fuse a secretion signal. The levels of expressed modified receptor are determined by the assays described herein.

EXAMPLE 11

Purification of Recombinant Modified NPY Receptor

Recombinantly produced modified receptor may be purified by a variety of procedures, including but not limited to antibody affinity chromatography.

Modified receptor antibody affinity columns are made by adding the anti-modified receptor antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1 M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents, and the cell culture supernatants or cell extracts containing solubilized modified receptor or modified

receptor subunits are slowly passed through the column. The column is then washed with phosphate-buffered saline (PBS) supplemented with detergents until the optical density (A280) falls to background; then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) supplemented with detergents. The purified modified receptor protein is then dialyzed against PBS.

EXAMPLE 12

Cloning and Expression of Modified NPY Receptor in Mammalian Cell System

A modified receptor is cloned into a mammalian expression vector. The mammalian expression vector is used to transform a mammalian cell line to produce a recombinant mammalian cell line. The recombinant mammalian cell line is cultivated under conditions that permit expression of the modified receptor. The recombinant mammalian cell line or membranes isolated from the recombinant mammalian cell line are used in assays to identify compounds that bind to the modified receptor.

EXAMPLE 13

Screening Assay

Recombinant cells containing DNA encoding a modified NPY receptor, membranes derived from the recombinant cells, or recombinant modified receptor preparations derived from the cells or membranes may be used to identify compounds that modulate modified NPY receptor activity. Modulation of such activity may occur at the level of DNA, RNA, protein or combinations thereof. One method of identifying compounds that modulate modified NPY receptor, comprises:

(a) mixing a test compound with a solution containing modified NPY receptor to form a mixture;

(b) measuring modified NPY receptor activity in the mixture; and

(c) comparing the modified NPY receptor activity of the mixture to a standard.

EXAMPLE 14

Formulation of Pharmaceutical Compositions

Compounds identified by the method of Example 13 are formulated into pharmaceutical compositions according to standard methods. The compounds or pharmaceutical compositions are used either alone or in combination with other compounds or compositions for the treatment of animals (including humans) in need of treatment. Conditions requiring treatment include but are not limited to obesity, regulation of apetite, congestive heart failure, diabetes, anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac and cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, and Huntington's, Alzheimer's and Parkinson's diseases.

EXAMPLE 15

Methods of Treatment

Animals (including humans) having a condition, the condition being characterized by factors selected from altered levels of neuropeptide Y, altered activities of neuropeptide Y, altered levels of neuropeptide Y receptor activity, altered neuropeptide Y receptor activity, and combinations thereof, are treated with compounds or derivatives of compounds identified by the screening method or pharmaceutical compositions comprising the compounds or derivatives of compounds identified by the screening method.

Animals (including humans) having a condition selected from obesity, diabetes, anxiety, hypertension, cocaine withdrawal, congestive heart failure, memory enhancement, cardiac vasospasm,

cerebral vasospasm, pheochromocytoma and ganglioneuroblastoma, Huntington's Disease, Alzheimer's Disease, Parkinson's disease, and combinations thereof, are treated with a therapeutically effective amount of compounds or derivatives of compounds identified by the screening method or pharmaceutical compositions comprising the compounds or derivatives of compounds identified by the screening method.

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptions, or modifications, as come within the scope of the following claims and its equivalents.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: STRADER, CATHERINE D.
CASCIERI, MARGARET A.
MACNEIL, DOUGLAS J.

(ii) TITLE OF INVENTION: MODIFIED NEUROPEPTIDE Y RECEPTORS

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: MARY A. APPOLLINA
(B) STREET: 126 EAST LINCOLN AVENUE
(C) CITY: RAHWAY
(D) STATE: NEW JERSEY
(E) COUNTRY: US
(F) ZIP: 07065-0900

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: APPOLLINA, MARY A.
(B) REGISTRATION NUMBER: 34,087
(C) REFERENCE/DOCKET NUMBER: 19339Y PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (908) 594-3462
(B) TELEFAX: (908) 594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile Tyr Ile Arg Leu Lys Arg Arg Asn Asn Met Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Met Arg Asp Asn Lys Tyr Arg Ser Ser Glu Thr Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Asn Ser Thr Leu Phe Ser Gln Val Glu Asn His Ser Asp Phe Leu
1 5 10 15

Val His Ser Asn Phe Ser Glu Lys Asn Ala Gln Leu Leu Ala Phe Glu
20 25 30

Asn Asp Asp Cys His Leu Pro Leu Ala Met Ile Phe Thr Leu Ala Leu
35 40 45

Ala Tyr Gly Ala Val Ile Ile Leu Gly Val Ser Gly Asn Leu Ala Leu
50 55 60

Ile Ile Ile Ile Leu Lys Gln Lys Glu Met Arg Asn Val Thr Asn Ile
65 70 75 80

Leu Ile Val Asn Leu Ser Phe Ser Asp Leu Leu Val Ala Ile Met Cys
85 90 95

Leu Pro Leu Thr Phe Val Tyr Thr Leu Met Asp His Trp Val Phe Gly
100 105 110

Glu Ala Met Cys Lys Leu Asn Pro Phe Val Gln Cys Val Ser Ile Thr
115 120 125

Val Ser Ile Phe Ser Leu Val Leu Ile Ala Val Glu Arg His Gln Leu
130 135 140

Ile Ile Asn Pro Arg Gly Trp Arg Pro Asn Asn Arg His Ala Tyr Val
145 150 155 160

Gly Ile Ala Val Ile Trp Val Leu Ala Val Ala Ser Ser Leu Pro Phe
165 170 175

Leu Ile Tyr Gln Val Met Thr Asp Glu Pro Phe Gln Asn Val Thr Leu
180 185 190

Asp Ala Tyr Lys Asp Lys Tyr Val Cys Phe Asp Gln Phe Pro Ser Asp
195 200 205

Ser His Arg Leu Ser Tyr Thr Thr Leu Leu Leu Val Leu Gln Tyr Phe
210 215 220

Gly Pro Leu Cys Phe Ile Phe Ile Cys Tyr Phe Lys Ile Tyr Ile Arg
225 230 235 240

Leu Lys Arg Arg Asn Asn Met Met Asp Lys Ser Glu Gly Arg Phe His
245 250 255

Ser Pro Asn Leu Gly Gln Val Glu Gln Asp Gly Arg Ser Gly His Gly
260 265 270

Leu Met Arg Asp Asn Lys Tyr Arg Ser Ser Glu Thr Lys Arg Ile Asn
275 280 285

Ile Met Leu Leu Ser Ile Val Val Ala Phe Ala Val Cys Trp Leu Pro
290 295 300

Leu Thr Ile Phe Asn Thr Val Phe Asp Trp Asn His Gln Ile Ile Ala
305 310 315 320

Thr Cys Asn His Asn Leu Leu Phe Leu Leu Cys His Leu Thr Ala Met
325 330 335

Ile Ser Thr Cys Val Asn Pro Ile Phe Tyr Gly Phe Leu Asn Lys Asn
340 345 350

Phe Gln Arg Asp Leu Gln Phe Phe Phe Asn Phe Cys Asp Phe Arg Ser
355 360 365

Arg Asp Asp Asp Tyr Glu Thr Ile Ala Met Ser Thr Met His Thr Asp
370 375 380

Val Ser Lys Thr Ser Leu Lys Gln Ala Ser Pro Val Ala Phe Lys Lys
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405 410

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Ile Arg Asp Ser Lys Tyr Arg Ser Ser Glu Thr Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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Ile Trp Ser Lys Leu Lys Asn His Val Ser Pro Gly
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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Asn Asp His Tyr His Gln Arg Arg Gln Lys Thr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Ala Ser Asp His Tyr His Gln Arg Arg His Lys Thr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ile Tyr Arg Arg Leu Gln Arg Gln Gly Arg Val Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ile Tyr Gln Arg Leu Gln Arg Gln Arg Arg Ala Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

His Lys Gly Thr Tyr Ser Leu Arg Ala Gly His Met Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

His Thr His Thr Cys Ser Ser Arg Val Gly Gln Met Lys
1 5 10

WHAT IS CLAIMED IS:

1. Isolated DNA encoding a modified neuropeptide Y receptor, the modified receptor being derived from a neuropeptide Y receptor having seven transmembrane domains and the modified neuropeptide Y receptor having deletions, replacements or additions in the third intracellular domain, or a functional derivative thereof.

2. The DNA of Claim 1 wherein the modified neuropeptide Y receptor is NPY4 receptor, or a functional derivative thereof.

3. The DNA of Claim 1 wherein the modified neuropeptide Y receptor is NPY1 receptor, or a functional derivative thereof.

4. The DNA of Claim 3 wherein the modified neuropeptide Y receptor is NPY1 receptor having deletions in the third intracellular domain, or a functional derivative thereof.

5. Isolated RNA encoded by the isolated DNA of Claim 1 or its complementary sequence.

6. The RNA of Claim 5 wherein the modified neuropeptide Y receptor is NPY4 receptor, or a functional derivative thereof.

7. The RNA of Claim 5 wherein the modified neuropeptide Y receptor is NPY1 receptor, or a functional derivative thereof.

8. The RNA of Claim 7 wherein the modified neuropeptide Y receptor is NPY1 receptor having deletions in the third intracellular domain, or a functional derivative thereof.

9. An expression vector comprising the isolated DNA of Claim 1.

10. The expression vector of Claim 9 comprising the isolated DNA which encodes NPY4 receptor, or a functional derivative thereof.

11. The expression vector of Claim 9 comprising the isolated DNA which encodes NPY1 receptor, or a functional derivative thereof.

12. The expression vector of Claim 11 comprising the isolated DNA which encodes NPY1 receptor having deletions in the third intracellular domain, or a functional derivative thereof.

13. A cell comprising the expression vector of Claim 9.

14. A process for production of a modified neuropeptide Y receptor, comprising:

a) transforming a host with the isolated DNA of Claim 1 to produce a recombinant host;

b) culturing the recombinant host under conditions which allow the production of modified neuropeptide Y receptor; and

c) recovering the modified neuropeptide Y receptor.

15. The modified neuropeptide Y receptor produced by the process of Claim 14.

16. An isolated and purified modified neuropeptide Y receptor, the modified receptor comprising a neuropeptide Y receptor having seven transmembrane domains wherein the modified receptor

has amino acids deleted from, replaced or added to the third intracellular domain, or a functional derivative thereof.

17. The modified receptor of Claim 16 wherein the modified neuropeptide Y receptor is NPY4 receptor, or a functional derivative thereof.

18. The modified receptor of Claim 16 wherein the modified neuropeptide Y receptor is NPY1 receptor, or a functional derivative thereof.

19. The modified receptor of Claim 18 wherein the modified neuropeptide Y receptor is NPY1 receptor having deletions in the third intracellular domain, or a functional derivative thereof.

20. A method of identifying compounds that modulate modified neuropeptide Y receptor activity, comprising:

(a) mixing a test compound with a solution containing modified neuropeptide Y receptor to form a mixture;

(b) measuring modified receptor activity in the mixture; and

(c) comparing the modified receptor activity of the mixture to a standard.

21. The method of Claim 20 wherein the modified neuropeptide Y receptor is NPY4 receptor.

22. The method of Claim 20 wherein the modified neuropeptide Y receptor is NPY1 receptor.

23. Antibody immunologically reactive with the modified receptor of Claim 16.

24. A method of making a modified neuropeptide Y receptor comprising:

(a) isolating DNA encoding a neuropeptide Y receptor;

(b) altering the DNA of step (a) by deleting at least one nucleotide from DNA encoding the third intracellular domain of the NPY receptor or disrupting the amphipathic helix at the N- or C-terminus of the third intracellular domain by replacement with nucleotides or addition of nucleotides coding for non-helical protein sequence;

(c) isolating the altered DNA;

(d) expressing the altered DNA; and

(e) recovering the modified receptor.

25. The modified receptor of Claim 24.

26. The method of Claim 24 wherein between six and thirteen nucleotides are deleted from DNA encoding the third intracellular domain of the neuropeptide Y receptor.

27. The modified receptors of Claim 26.

28. The isolated DNA of Claim 1 wherein the modified neuropeptide Y receptor is selected from the group consisting of:

(a) D(234-241)NPY1 receptor; and

(b) D(247-259)NPY1 receptor.

29. The isolated and purified receptor of Claim 16 wherein the modified neuropeptide Y receptor is selected from the group consisting of:

(a) D(234-241)NPY1 receptor; and

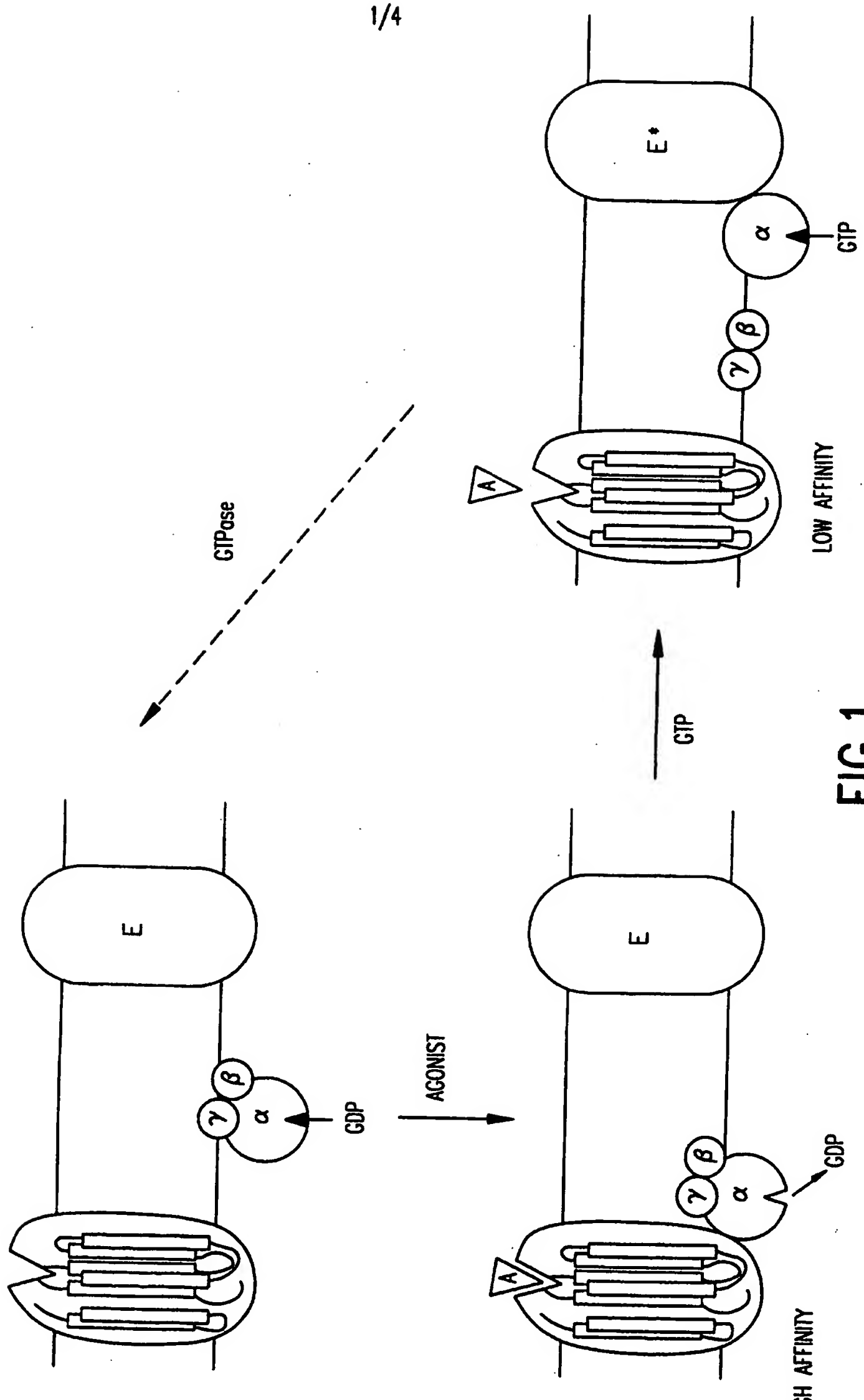
(b) D(247-259)NPY1 receptor.

30. The isolated DNA of Claim 1 encoding a member of the family of NPY receptors having deletions, replacements or additions of the N or C terminal portions of the third intracellular loop, such that the modified receptor binds agonists in the high affinity state regardless of the presence or absence of G proteins.

31. The isolated DNA of Claim 30 encoding a member of the family of NPY receptors having deletions of the N or C terminal portions of the third intracellular loop, such that the modified receptor binds agonists in the high affinity state regardless of the presence or absence of G proteins.

32. The isolated and purified receptor of Claim 16 wherein the modified neuropeptide Y receptor is a member of a family of NPY receptors having deletions, replacements or additions of the N or C terminal portions of the third intracellular loop, such that the modified receptor binds agonists in the high affinity state regardless of the presence or absence of G proteins.

33. The isolated and purified receptor of Claim 32 wherein the modified neuropeptide Y receptor is a member of a family of NPY receptors having deletions of the N or C terminal portions of the third intracellular loop, such that the modified receptor binds agonists in the high affinity state regardless of the presence or absence of G proteins.


E*
α
GTP
β
γ
A
LOW AFFINITY
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AGONIST
GDP
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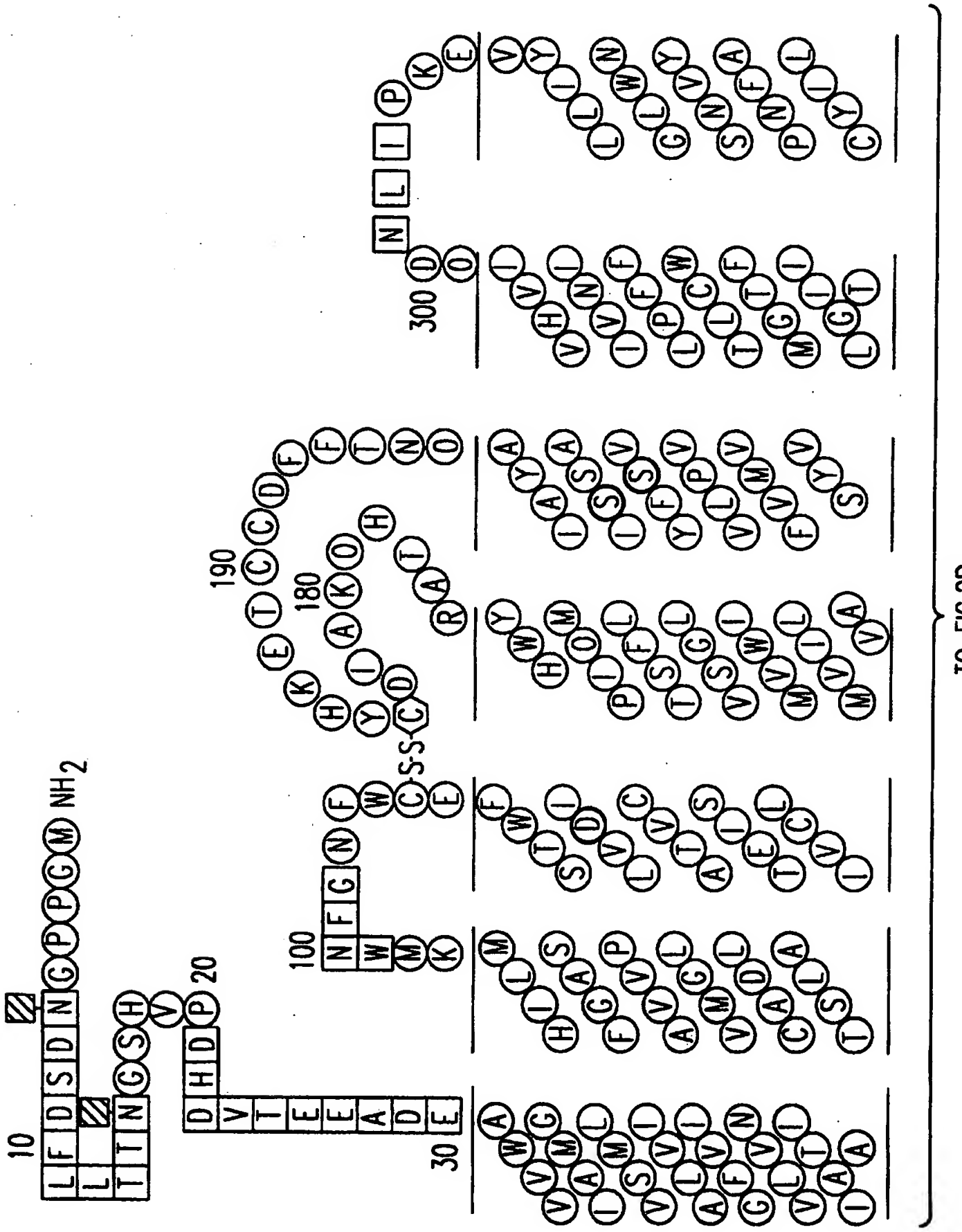
HIGH AFFINITY

FIG.1

FIG.2A



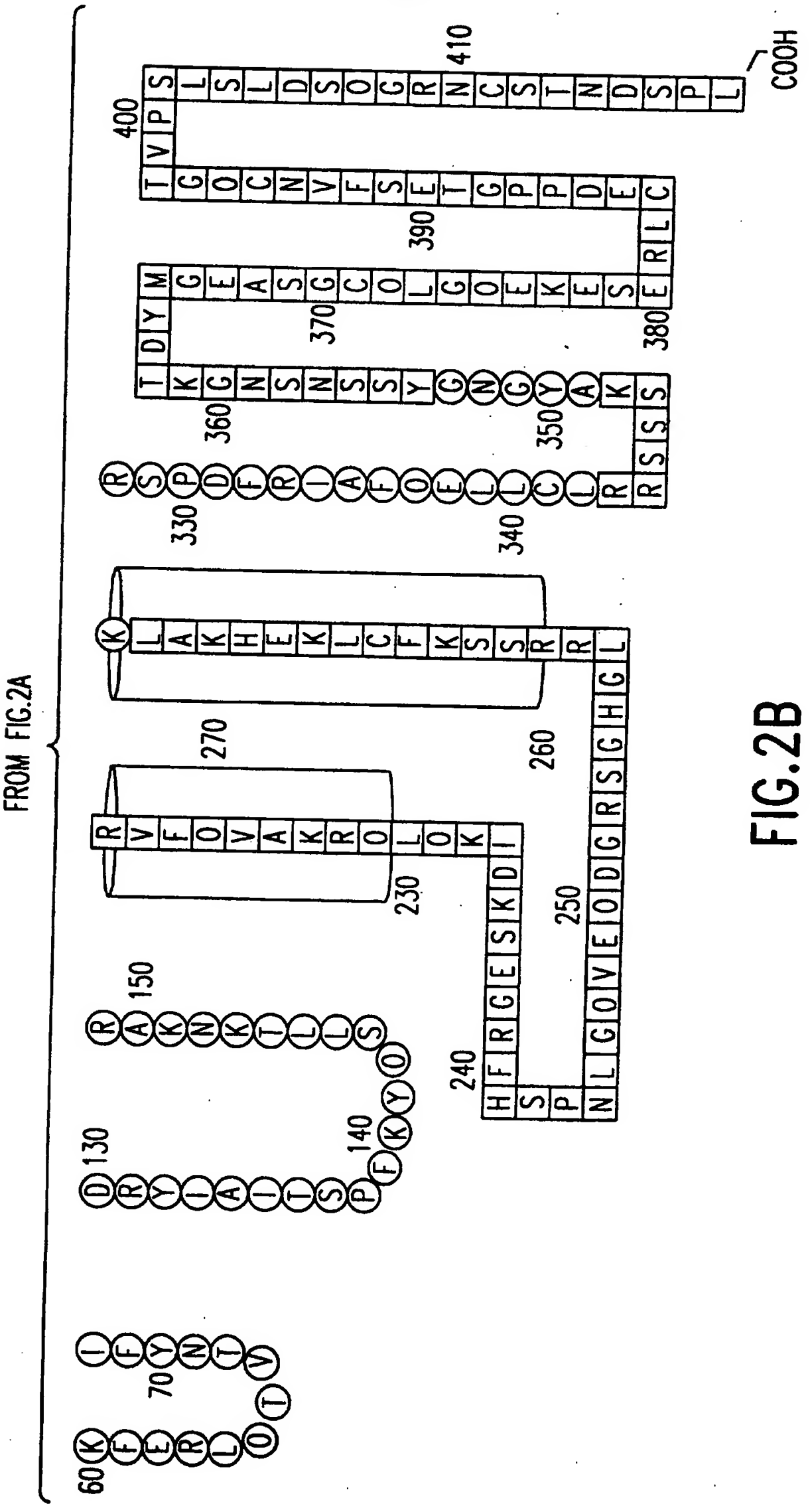

FROM FIG.2A
60 K F E R L O T I
70 Y N T V
D R Y I A I T S P F K Y O S
130 140
R A K N K T L L S
150
R V F O V A K R O L O K
230
270
L A K H E K L C F K S S R R
260
H F R G E S K D I
240
S P
250
N L G O V E O D G R S G H G L
400 T V P S
410 L S L D S O G R N C S T N D S P L
COOH
T G O C N V F S E T G P P D E C
390
T D Y M G E A S G C O L G O E K E S E R L C
370
380
T K G N S N S S Y G N G Y A K
360
350
R S P D F R I A F O E J L C J R R S S K

FIG.2B

```
1   .....MGPPGNDSDFLLTTNGSHVPDHDVTEERDE.....AWVVGMAILM  40
1   MNSTLFSQVENHS...VHSNFSEKNAQLLAFENDDCHLPLAMIFTLALAY  47

41  SVIVLAIVFGNVLVITAIAKFERLQTVTNYFITSLACADLVMGLAVVPFG  90
48  GAVIILGVSGNLALIIIILKQKEMRNVTNILIVNLSFSDLLVAIMCLPLT  97

91  ASHILMKMWNFGNFWCEFWTSIDVLCVTASIETLCVIAVDRYIAITSPFK 140
98  FVYTLMDHWVFGEAMCKLNPFVQCVSITVSIFSLVLIAVERHQLIINPRG 147

141 YQSLLTKNKARMVILMVWIVSGLTSFLPIQMHWYRATHQKAIDCY.HKET 189
148 WRPNNRHAYVGIAVIWVLAVASSLPFLIYQVMTDEPFQNVTLDAYKDKYV 197

190 CCDFFTNQ....AYAIASSIVSFYVPLVVMVFVYSRVFQVAKRQLQKIDK 235
198 CFDQFPSDSHRLSYTTLLLVLQYFGPLCFIFICYFKIYIRLKRRNNMMDK 247

236 SEGRFHSPNLGQVEQDGRSGHGLRRSSKFCLKEHKALKTLGI.IMGTFTL 284
248 .....MRDNKYRSSETKRINIMLLSIVVAFAV 274

285 CWLPFFIVNIVHVIQDNLIP....KEVYILLNWLGYVNSAFNPLIYC.RS 329
275 CWLPLTIFNTVFDWNHQIIATCNHNLLFLLCHLTAMISTCVNPIFYGFLN 324

330 PDFRIAFQELLCLRRSSSKAYGNGYSSNSNGKTDY....MGEASGCQL.. 373
325 KNFQRDLQFFFNFCDFRSRDDDYETIAMSTMHTDVSKTSLKQASPVAFKK 374

374 .GQEKESERLCEDPPGTESFVNCQGTVPSLSLDSQGRNCSTNDSPLX 419
375 INNNDDNEKIX..................................... 385
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FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14377

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 69.1; 70.1, 240.1, 320.1; 530/350; 536/23.5; 930/10; 935/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, EMBASE, BIOSIS, MEDLINE, CA, WPIDS

search terms: neuropeptide, Y, YY, NPY, receptor#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Biological Chemistry, Volume 267, No. 16, issued 05 June 1992, LARHAMMAR et al., "Cloning and Functional Expression of a Human Neuropeptide Y/Peptide YY Receptor of the Y1 Type", pages 10935-10938, see entire document.	1-22, 24-33
Y	The Journal of Biological Chemistry, Volume 268, No. 22, issued 05 August 1993, REN et al., "Constitutively Active Mutants of the α2-Adrenergic Receptor", pages 16483-16487, see pages 16483-16486.	1-22, 24-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 31 JANUARY 1996	Date of mailing of the international search report 21 FEB 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN GUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14377

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Biological Chemistry, Volume 262, No. 34, issued 05 December 1987, STRADER et al., "Mutations That Uncouple the β-Adrenergic Receptor from Gs and Increase Agonist Affinity", pages 16439-16443, see pages 16441-16443.	1-22, 24-33
Y	The Journal of Biological Chemistry, Volume 263, No. 31, issued 05 November 1988, O'DOWED et al., "Site -directed Mutagenesis of the Cytoplasmic Domains of the Human β2-Adrenergic Receptor", pages 15985-15992, see pages 15985-15988, 15990.	1-22, 24-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14377

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22, 24-33

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14377

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C07K 1/00, 14/00; C12N 5/00, 15/00, 15/09, 15/11, 15/12, 15/63; C12P 21/04, 21/06

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.2, 69.1, 70.1, 240.1, 320.1; 530/350; 536/23.5; 930/10; 935/1

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-22 and 24-33 drawn to nucleic acids, vectors, host cells, proteins, method of making proteins, and a method of using the proteins in receptor binding.

Group II, claim(s) 23, drawn to an antibody.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group II is drawn to an antibody and therefore does not share the special technical feature of nucleic acids encoding for neuropeptide Y (NPY) receptors. Furthermore, an antibody is structurally and functionally different and distinct from either nucleic acids encoding the NPY receptor or the amino acid sequence consisting of the NPY receptor. The NPY receptor may bind ligands to the receptor, but an antibody to the receptor binds to the receptor itself, and does not bind to ligands to the receptor. An antibody to the NPY receptor is not used in or produced by the methods of Group I.Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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International Bureau

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(71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US).

(72) Inventors: RACIE, Lisa, A.; 124 School Street, Acton, MA 01720 (US). LAVALLIE, Edward, R.; 90 Green Meadow Drive, Tewksbury, MA 01876 (US). MCCOY, John, M.; 56 Howard Street, Reading, MA 01867 (US).

(74) Agent: BROWN, Scott, A.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US).

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Published
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(54) Title: BETA-CHEMOKINE, H1305 (MCP-2)

(57) Abstract

Polynucleotides encoding H1305 and related proteins are disclosed. H1305 proteins and methods for their production are also disclosed.

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BETA-CHEMOKINE, H1305 (MCP-2)

Field of the Invention

The present invention relates to H1305 proteins, nucleic acids encoding such proteins, and methods of treatment using such proteins.

Background of the Invention

Chemokines are a subclass of cytokines which cause the directed migration or chemotaxis of particular cell populations either to or away from higher concentrations of the chemokine. Many chemokines have been identified which cause migration of major blood cell populations. These factors may be useful for directing the migration of cell populations to areas of desired action or away from areas of unwanted action. It would, therefore, be desirable to identify new chemokines and polynucleotides encoding them.

Summary of the Invention

In developing the present invention, methods were employed which selectively identify polynucleotides which encode secreted proteins. One such polynucleotide was isolated which encodes "H1305." In accordance with the present invention, polynucleotides encoding H1305 and active fragments thereof are disclosed. "H1305" is used throughout the present specification to refer to both proteins and polynucleotides encoding those proteins and to refer to proteins and polynucleotides from all mammalian species.

In certain embodiments, the present invention provides an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 47 to nucleotide 373;

(b) a nucleotide sequence capable of hybridizing to a nucleic acid sequence specified in (a);

(c) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code;

(d) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:2; and

(e) an allelic variant of the nucleotide sequence specified in (a).

Polynucleotides comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 47 to nucleotide 373 are particularly preferred. Preferably, the polynucleotide of the invention encodes a protein having H1305 activity. In other embodiments the polynucleotide is operably linked to an expression control sequence.

Host cells transformed with the polynucleotides of the invention are also provided, including mammalian cells.

Processes are also provided for producing a H1305 protein, said processes comprising:

(a) growing a culture of the host cell of the invention in a suitable culture medium; and

(b) purifying the H1305 protein from the culture.

Isolated H1305 protein is also provided which comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2; and

(b) fragments of (a) having H1305 activity.

Proteins comprising the amino acid sequence of SEQ ID NO:2 are particularly preferred. Preferably, the protein has H1305 activity. Pharmaceuticals composition comprising a H1305 protein of the invention and a pharmaceutically acceptable carrier are also provided.

Compositions are also disclosed which comprise an antibody which specifically reacts with a H1305 protein of the invention.

Methods of treating a mammalian subject are also provided which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a H1305 protein.

Detailed Description of Preferred Embodiments

The inventors of the present application have identified and provided a polynucleotide encoding a H1305 protein. SEQ ID NO:1 provides the nucleotide sequence of a cDNA encoding the H1305 protein. SEQ ID NO:2 provides the amino acid sequence of the H1305 protein predicted from the DNA coding sequence. The amino acid sequence of H1305 contains known chemokine sequence motifs and shows homology with known chemokines.

Forms of H1305 protein of less than full length are encompassed within the present invention and may be produced by expressing a corresponding fragment of the polynucleotide encoding the H1305 protein (SEQ ID NO:1). These corresponding polynucleotide fragments are also part of the present invention. Modified polynucleotides as described above may be made by standard molecular biology techniques. including site-directed mutagenesis methods which are known in the art or by the polymerase chain reaction using appropriate oligonucleotide primers. A mature form of the H1305 protein, if it differs from the full-length sequence of SEQ ID NO:2, can be produced by expression of the full-length polynucleotide in an appropriate cell line.

For the purposes of the present invention, a protein has "H1305 activity" if it either (1) displays chemoattractant or chemotactic activity in a chemoattraction or chemotaxis assay (preferably an assay in which the corresponding species full-length H1305 is active), or (2) displays biological activity in a factor-dependent cell proliferation assay (preferably an assay in which the corresponding species full-length H1305 is active), or (3) displays activity in the induction of lymphokine production or effector function in an immune cell functional assay. Examples of effector function include, without limitation, tumoricidal activity, granule release, adhesion molecule expression, and the like. Activity may be monitored using assays known in the art. Chemoattractant or chemotactic activity can also be measured *in vivo* by injecting protein at a particular site and performing a histological examination of the cell types that migrate to the site of injection.

H1305 protein or fragments thereof having H1305 activity may be fused to carrier molecules such as immunoglobulins. For example, H1305 protein may be fused through "linker" sequences to the Fc portion of an immunoglobulin.

The invention also encompasses allelic variations of the nucleotide sequence as set forth in SEQ ID NO:1, that is, naturally-occurring alternative forms of the isolated polynucleotide of SEQ ID NO:1 which also encode H1305 or H1305 proteins having H1305 activity. Also included in the invention are isolated polynucleotides which hybridize to the nucleotide sequence set forth in SEQ ID NO:1 under highly stringent (e.g., 0.2xSSC at 65°C). stringent (e.g. 4xSSC at 65°C or 50% formamide and 4xSSC at 42°C), or relaxed (e.g., 4xSSC at 50°C or 30-40% formamide and 4xSSC at 42°C) conditions. Isolated polynucleotides which encode H1305 protein but which differ from the nucleotide sequence set forth in SEQ ID NO:1 by virtue of the degeneracy of the genetic code are also encompassed by the present invention. Variations in the nucleotide sequence as set forth in SEQ ID NO:1 which are caused by point mutations or by induced modifications which enhance H1305 activity, half-life or production level are also included in the invention.

Polynucleotides encoding H1305 proteins of other animal species, particularly mammalian species, can be obtained by using portions of SEQ ID NO:1 as a probe of a DNA library made from appropriate sources for such other species.

The isolated polynucleotides of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the H1305 protein recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated polynucleotide of the invention and the expression control sequence, in such a way that the H1305 protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the H1305 protein. Any cell type capable of expressing functional H1305 protein may be used. Suitable mammalian host cells include, for example, monkey COS

cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK, Rat2, BaF3, 32D, FDCP-1, PC12 or C2C12 cells.

The H1305 protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference.

Alternatively, the H1305 protein may be produced in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Suitable yeast strains include Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous proteins.

The H1305 protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide sequence encoding the H1305 protein.

The H1305 protein of the invention may be prepared by growing a culture of transformed host cells under culture conditions necessary to express the desired protein. The resulting expressed protein may then be purified from the culture medium or cell extracts. The H1305 protein of the invention can be purified from conditioned media.

The H1305 protein can be purified using methods known to those skilled in the art. For example, the H1305 protein of the invention can be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration

step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the H1305 protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography. Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the H1305 protein. Some or all of the foregoing purification steps, in various combinations or with other known methods, can also be employed to provide a substantially purified isolated recombinant protein.

Preferably, the H1305 protein is purified so that it is substantially free of other mammalian proteins.

It is believed that H1305, active fragments and variants thereof, and H1305 related proteins (collectively "H1305 proteins") possess chemokine activities. Therefore, H1305 and H1305 related proteins may have an effect on chemotaxis or migration of blood cells, including without limitation eosinophils, basophils, dendritic cells, natural killer cells, neutrophils, monocytes, T cells and mast cells. A protein or peptide has "chemotactic activity," as used herein, if it can stimulate, directly or indirectly, the directed orientation or movement of cells, including myeloid and lymphoid cells. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells. Whether a particular protein or peptide has chemotactic activity for cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

H1305 proteins may also be useful for inhibition of viral replication, including without limitation replication of HIV.

Isolated H1305 proteins, purified from cells or recombinantly produced, may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may contain, in addition to H1305 protein and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, γ-IFN, stem cell factor, and erythropoietin. The pharmaceutical composition may contain thrombolytic or anti-thrombotic factors such as plasminogen activator and Factor VIII. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with H1305 protein, or to minimize side effects caused by the H1305 protein. Conversely, H1305 protein may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which H1305 protein is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for

example, in U.S. Patent No. 4.235,871; U.S. Patent No. 4.501,728; U.S. Patent No. 4.837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, e.g., amelioration of symptoms of, healing of, or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention. a therapeutically effective amount of H1305 protein is administered to a mammal. H1305 protein may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines, other hematopoietic factors or vaccine components (such as antigens or other adjuvants), H1305 protein may be administered either simultaneously with the cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering H1305 protein in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of H1305 protein used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of H1305 protein is administered orally, H1305 protein will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the

invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% H1305 protein, and preferably from about 25 to 90% H1305 protein. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of H1305 protein, and preferably from about 1 to 50% H1305 protein.

When a therapeutically effective amount of H1305 protein is administered by intravenous, cutaneous or subcutaneous injection, H1305 protein will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to H1305 protein an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

The amount of H1305 protein in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of H1305 protein with which to treat each individual patient. Initially, the attending physician will administer low doses of H1305 protein and observe the patient's response. Larger doses of H1305 protein may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not generally increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 μg to about

100 mg of H1305 protein per kg body weight, preferably about 0.1 μg to about 10 mg of H1305 protein per kg body weight, more preferably about 0.1 μg to about 100 μg of H1305 protein per kg body weight, most preferably about 0.1 μg to about 10 μg of H1305 protein per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the H1305 protein will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

H1305 proteins may also be useful for treatment of wounds. In such applications, the protein may be administered as described above, if appropriate, or may be applied in other suitable forms, such as by topical administration in the form of a solution, suspension, ointment, salve, compress and the like.

H1305 protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the H1305 protein and which may inhibit H1305 binding to its receptor. Such antibodies are also useful for performing diagnostics assays for H1305 in accordance with known methods. Such antibodies may be obtained using the entire H1305 protein as an immunogen, or by using fragments of H1305 protein. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, for example, as in R.P. Merrifield, J.Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, et al., FEBS Lett. 211, 10 (1987).

Neutralizing or non-neutralizing antibodies (preferably monoclonal antibodies) binding to H1305 protein may also be useful therapeutics for certain tumors and also in the treatment of conditions described above. These neutralizing

monoclonal antibodies are capable of blocking the ligand binding to the H1305 protein or may promote clearance of protein from the patient.

Example

Isolation of H1305 cDNA

A partial clone for H1305 was isolated from a cDNA library made from RNA isolated from stimulated human peripheral blood mononuclear cells using methods which are selective for secreted proteins. Sequence from this partial clone was then used to identify a full-length clone from a PBMC library. Comparison of this sequence to the sequence of the original partial clone confirmed identity and that the isolated cDNA was full-length. The full-length clone (H1305, SEQ ID NO:1) was deposited with the American Type Culture Collection on December 13, 1995 and assigned accession number ATCC 69968.

All patent and literature references cited herein are incorporated by reference as if fully set forth.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Racie, Lisa A.
LaVallie, Edward R.
McCoy, John

(ii) TITLE OF INVENTION: Chemokine H1305

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genetics Institute, Inc.
(B) STREET: 87 CambridgePark Drive
(C) CITY: Cambridge
(D) STATE: Massachusetts
(E) COUNTRY: USA
(F) ZIP: 02140

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Brown, Scott A.
(B) REGISTRATION NUMBER: 32,724
(C) REFERENCE/DOCKET NUMBER: GI5265

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 498-8224
(B) TELEFAX: (617) 876-5851

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 47..373

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCGGCC AAAGAGGCTA GAACAACCCA GAAACCTTCA CCTCTC ATG CTG AAG 55
Met Leu Lys
1

```
CTC ACA CCC TTG CCC TCC AAG ATG AAG GTT TCT GCA GCG CTT CTG TGC        103
Leu Thr Pro Leu Pro Ser Lys Met Lys Val Ser Ala Ala Leu Leu Cys
  5                  10                  15

CTG CTG CTC ATG GCA GCC ACT TTC AGC CCT CAG GGA CTT GCT CAG CCA        151
Leu Leu Leu Met Ala Ala Thr Phe Ser Pro Gln Gly Leu Ala Gln Pro
 20                  25                  30                  35

GAT TCA GTT TCC ATT CCA ATC ACC TGC TGC TTT AAC GTG ATC AAT AGG        199
Asp Ser Val Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile Asn Arg
             40                  45                  50

AAA ATT CCT ATC CAG AGG CTG GAG AGC TAC ACA AGA ATC ACC AAC ATC        247
Lys Ile Pro Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr Asn Ile
         55                  60                  65

CAA TGT CCC AAG GAA GCT GTG ATC TTC AAG ACC CAA CGG GGC AAG GAG        295
Gln Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Gln Arg Gly Lys Glu
     70                  75                  80

GTC TGT GCT GAC CCC AAG GAG AGA TGG GTC AGG GAT TCC ATG AAG CAT        343
Val Cys Ala Asp Pro Lys Glu Arg Trp Val Arg Asp Ser Met Lys His
  85                  90                  95

CTG GAC CAA ATA TTT CAA AAT CTG AAG CCA TGAGCCTTCA TACATGGACT          393
Leu Asp Gln Ile Phe Gln Asn Leu Lys Pro
100                 105

GAGAGTCAGA GCTTGAAG                                                    411
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Leu Lys Leu Thr Pro Leu Pro Ser Lys Met Lys Val Ser Ala Ala
 1               5                  10                  15

Leu Leu Cys Leu Leu Leu Met Ala Ala Thr Phe Ser Pro Gln Gly Leu
            20                  25                  30

Ala Gln Pro Asp Ser Val Ser Ile Pro Ile Thr Cys Cys Phe Asn Val
        35                  40                  45

Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile
    50                  55                  60

Thr Asn Ile Gln Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Gln Arg
65                  70                  75                  80

Gly Lys Glu Val Cys Ala Asp Pro Lys Glu Arg Trp Val Arg Asp Ser
                85                  90                  95

Met Lys His Leu Asp Gln Ile Phe Gln Asn Leu Lys Pro
            100                 105
```

What is claimed is:

1. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 47 to nucleotide 373;

(b) a nucleotide sequence capable of hybridizing to a nucleic acid sequence specified in (a);

(c) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code;

(d) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:2; and

(e) an allelic variant of the nucleotide sequence specified in (a).

2. The polynucleotide of claim 1 wherein said nucleotide sequence encodes a protein having H1305 activity.

3. The polynucleotide of claim 1 wherein said nucleotide sequence is operably linked to an expression control sequence.

4. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 47 to nucleotide 373.

5. A host cell transformed with the polynucleotide of claim 3.

6. The host cell of claim 5, wherein said cell is a mammalian cell.

7. A process for producing a H1305 protein, said process comprising:

(a) growing a culture of the host cell of claim 5 in a suitable culture medium; and

(b) purifying the H1305 protein from the culture.

8. An isolated H1305 protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2; and

(b) fragments of (a) having H1305 activity.

9. The protein of claim 8 comprising the amino acid sequence of SEQ ID NO:2.

10. A pharmaceutical composition comprising a H1305 protein of claim 8 and a pharmaceutically acceptable carrier.

11. A H1305 protein produced according to the process of claim 7.

12. A composition comprising an antibody which specifically reacts with a H1305 protein of claim 8.

13. A method of treating a mammalian subject comprising administering a therapeutically effective amount of a composition of claim 10.

INTERNATIONAL SEARCH REPORT

International Application No
PC./US 97/00379

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C07K14/52 A61K38/19 C07K16/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL IMMUNOLOGY, vol. 1, 1989, pages 388-399, XP000673182 CHANG, H. ET AL.: "Cloning and expression of a gamma-interferon-inducible gene in monocytes: a new member of a cytokine gene family" see figure 7 ---	1-3,5,7, 8,11
X	DNA AND CELL BIOLOGY, vol. 13, January 1994, pages 1-8, XP000673202 WEMPE, F. ET AL.: "Cloning of the gene for bovine monocyte chemoattractant protein-2" see figures 1-3 --- -/--	1-3,5,7, 8,11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 1997	02. 05. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Andres, S

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INTERNATIONAL SEARCH REPORT

Inter "ional Application No
PC:/US 97/00379

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF EXPERIMENTAL MEDICINE, vol. 176, July 1992, pages 59-65, XP000673185 VAN DAMME, J. ET AL.: "Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonginig to the chemokine family" see figure 3 ---	8,11,12
X	JOURNAL OF LEUKOCYTE BIOLOGY, vol. 57, no. 5, May 1995, pages 703-711, XP000605180 CLARK-LEWIS I ET AL: "STRUCTURE-ACTIVITY RELATIONSHIPS OF CHEMOKINES" see figure 1 ---	8,11
P,X	WO 96 40923 A (ICOS CORP) 19 December 1996 see page 78, line 3 - line 22 see figure 1 ---	1-13
P,X	WO 96 32481 A (INCYTE PHARMA INC) 17 October 1996 see SEQ ID 6 -----	1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/00379

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640923 A	19-12-96	AU 6172496 A	30-12-96
		CA 2196691 A	19-12-96
WO 9632481 A	17-10-96	AU 5543496 A	30-10-96